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TOWNSEND and TOWNSEND and CREW LLP

By: /Aaron Hokamura/

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of:

Thomas J. Schall et al.

Application No.: 10/001,221

Filed: October 30, 2001

For: COMPOSITIONS FOR INDUCING  
AN IMMUNE RESPONSE

Confirmation No. 2004

Examiner: Canella, Karen A.

Technology Center/Art Unit: 1643

APPELLANTS' AMENDED BRIEF  
UNDER 37 CFR § 41.37

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Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Sir:

Further to the Notice of Appeal filed on June 26, 2006 for the above-referenced application and in response to the Notification of Non-Compliant Appeal Brief (37 CFR 41.37) mailed January 11, 2007, Appellants submit this Amended Brief on Appeal.

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## **1. REAL PARTY IN INTEREST**

ChemoCentryx, Inc.

## **2. RELATED APPEALS AND INTERFERENCES**

None.

## **3. STATUS OF CLAIMS**

Claims 69-72, 75, 79-93 and 97-106 stand rejected and are appealed. Claims 76-78 and 94-96 are withdrawn from consideration. Claims 1-68 and 73-74 are canceled.

## **4. STATUS OF AMENDMENTS**

The Amendment After Final filed April 3, 2006 was entered.

## **5. SUMMARY OF CLAIMED SUBJECT MATTER**

Independent claim 69 is directed to a pharmaceutical composition comprising an antigen-presenting cell (APC) chemotaxin, viral murine cytomegalovirus chemokine 2 (vMCK-2), and an antigen. APC chemotaxins are chemotactic for, *i.e.*, attract) immature and/or mature dendritic cells. When the claimed pharmaceutical composition is administered to a subject, the vMCK-2 can enhance the immune response, *i.e.*, antibody-mediated and/or cell-mediated response, to the antigen. Compositions comprising vMCK-2 are described in the specification at, *e.g.*, page 30, lines 5 to 32. The ability of vMCK-2 to attract mononuclear cells after intradermal injection of Rhesus monkeys is described in Examples 4 and 5 (page 65, line 1 to page 67, line 16). Tables 6 and 7 show that the injection of vMCK-2 caused substantial mononuclear cell infiltration, *i.e.*, primarily neutrophils and APCs, including macrophages and dendritic cells, at 24 and 48 hours after injection. The ability of vMCK-2 to enhance the antibody-mediated response to an antigen in Rhesus monkey is described in Example 6 (page 68, line 1 to page 70,

line 12). Table 8 shows that Rhesus monkeys injected with ovalbumin, an adjuvant and vMCK-2 developed a significant anti-ovalbumin antibody response.

Independent claim 89 is directed to a pharmaceutical composition comprising an APC chemotaxin, murine C10 (mC10), and an antigen. When the claimed pharmaceutical composition is administered to a subject, the mC10 can enhance the immune response, *i.e.*, antibody-mediated and/or cell-mediated response, to the antigen. Compositions comprising mC10 are described in the specification at, *e.g.*, page 30, lines 5 to 32. The ability of mC10 to attract dendritic cells after intradermal injection of mice is described in Example 2 (page 61, line 1 to page 62, line 2). Table 4 shows that mice injected with mC10 caused substantial dendritic cell infiltration to the site of injection after 72 hours. The ability of mC10 to induce mononuclear cell infiltration in the dermis after intradermal injection of Rhesus monkeys is described in Examples 3 to 5 (page 62, line 3 to page 67, line 16). Tables 5 to 7 show that injection of mC10 caused substantial mononuclear cell infiltration, *i.e.*, primarily APCs, including macrophages and dendritic cells, but few neutrophils, at 24, 48, 72 and 96 hours after injection; little or no mononuclear cell infiltration was observed after injection of high concentrations of mC10. The ability of mC10 to enhance the antibody-mediated response to ovalbumin in Rhesus monkey is described in Example 6 (page 68, line 1 to page 70, line 12). Table 8 shows that Rhesus monkeys injected with ovalbumin, adjuvant and mC10 developed a significant anti-ovalbumin antibody response.

Thus, the claimed pharmaceutical compositions comprise an antigen and an APC chemotaxin, either mouse virus encoded vMCK-2 or murine mC10, which has the ability to bind to and activate primate chemokine receptors on APCs, *e.g.*, macrophages and dendritic cells; to attract unexpectedly high numbers of APCs to the site of injection, and to significantly enhance the immune response to the co-administered antigen. Appellants' results are unexpected and particularly surprising because the skilled artisan could not have predicted that vMCK-2 and mC10 would have such high chemoattractant activity for dendritic cells and to be able to exert biological activity across species, *i.e.*, rodent-primate, lines.

## **6. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL**

Issue 1: Whether claims 89-93 and 97-106 would have been obvious over Kedar et al., *Adv. Cancer Res.* 59:245-322, 1992 ("Kedar") in view of Bystrytn, WO 98/33520 ("Bystrytn"), Mohamadzadeh et al., *Arch. Dermatol. Res.* 289:435-439, 1997 ("Mohamadzadeh") and Orlofsky et al., *Cytokine* 12:220-228, 2000 ("Orlofsky") under 35 U.S.C. § 103(a).

Issue 2: Whether claims 69-72, 75 and 79-88 would have been obvious over Kedar, in view of Bystrytn and Saederup et al., *Proc. Natl. Acad. Sci. USA* 96:10881-10886, 1999 ("Saederup") under 35 U.S.C. § 103(a).

## **7. ARGUMENT**

### **A. Issue 1: Claims 89-93 and 97-106 Not Obvious Over Kedar in View of Bystrytn, Mohamadzadeh and Orlofsky**

#### **1.1 The Examiner's Rationale**

The Examiner's rationale is set forth on pages 3-5 of the Final Office Action mailed January 3, 2006.

The Examiner cites Kedar as discussing cancer immunotherapy methods. According to the Examiner, Kedar teaches that: (1) antigenicity of a tumor and capacity to mobilize a T-cell response are required for successful immunotherapy; (2) infiltrating macrophage, neutrophils and eosinophils, which are present in regressing tumors, are mobilized by lymphokines released from antigen-specific T-cells; (3) infiltrating cells contribute to the therapeutic effect; (4) stealth liposomes can achieve prolonged circulation time and enhanced accumulation in tumors; (5) administration of biological modifiers, such as cytokines, by encapsulation in liposomes bypasses the need for continuous infusion or frequent bolus administration of the biological response modifier; (6) tumor antigens encapsulated in liposomes can improve immunogenicity of the tumor antigen in humans; (7) administration of the tumor antigen together with cytokines and improved adjuvants increases anti-tumor efficacy in

experimental animals; and (8) treatment with combinations of sub-toxic doses of cytokines with different activities may improve therapeutic index. The Examiner acknowledges that Kedar does not specifically teach mC10 as a biological response modifier, or an encapsulated liposome comprising a chemokine, adjuvant and a tumor antigen.

The Examiner cites Bystryn as discussing pH sensitive liposomes. According to the Examiner, Bystryn teaches: (1) pH sensitive liposome encapsulated vaccines containing immunomodulators, including cytokines such as IL-1, IL-2, IL-6, IL-12 and GM-CSF; (2) administration of encapsulated vaccines by various routes; and (3) pH sensitive liposomes are taken up by APCs.

The Examiner cites Mohamadzadeh as discussing mC10. According to the Examiner, Mohamadzadeh teaches that dendritic cells and Langerhan's cells: (1) are sources of mC10, which recruits T-cells, and cytokines, which are involved in the initiation of inflammatory events; and (2) can process and present protein antigens and induce primary T-cell responses.

The Examiner cites Orlofsky as discussing mC10. According to the Examiner, Orlofsky teaches that: (1) Th2-type immune reactions are modulated by mC10; (2) subsequent development of the Th2 response is ineffective in suppressing mC10 expression; (3) mC10 is chemotactic for macrophages, and for T and B lymphocytes; and (4) mC10 either maintains modes of cellular inactivity previously initiated by transient chemokines, or specifically attracts one or more T-cell subsets.

The Examiner alleges that it would have been obvious to use mC10 as an immunomodulator in the stealth liposomes taught by Kedar, and to combine mC10 with a tumor antigen, an additional chemokine and an adjuvant in the liposomes. The Examiner alleges that the skilled artisan would have been motivated by the teachings of Kedar, on accumulation of stealth liposomes at the tumor site, Orlofsky, on maintenance of the Th2 response by mC10, and Orlofsky and Mohamadzadeh, on recruitment of T cells and the involvement of cytokines in the inflammatory response.

The motivation asserted by the Examiner to combine liposome encapsulated mC10 with a tumor antigen is that Kedar teaches that encapsulation of tumor antigens within liposomes can improve immunogenicity. The motivation asserted by the Examiner to combine mC10 with an additional chemokine is to exert an additive or synergistic effect. The motivation asserted by the Examiner to prepare sterile preparations of the liposome encapsulated pharmaceuticals is to preserve shelf life of the pharmaceuticals, and to prevent contamination.

## 1.2 Summary of the References

### 1.2.1 Kedar

Kedar reviews cancer immunotherapy and provides a variety of suggestions to improve the efficacy and safety of cancer immunotherapy. In particular, Kedar discusses the use of a wide variety of cytokines, alone or in combination with other biological response modifiers, antigens and/or immune cells, in cancer immunotherapy in humans and in animal model systems. Kedar does not discuss or even mention the use of chemokines generally, or mC10 specifically, as immunomodulators in cancer immunotherapy.

### 1.2.2 Bystryn

Bystryn discusses vaccine compositions having an antigen, an immunomodulator and a pH sensitive liposome as carrier. Bystryn lists several cytokines, including IL-1, IL-2, IL-6, IL-12 and GM-CSF, as immunomodulators that may enhance and amplify immune responses induced by an antigen. Bystryn does not discuss or even mention the use of chemokines generally, or mC10 specifically, as immunomodulators in immunotherapy.

### 1.2.3 Mohamadzadeh

Mohamadzadeh discusses dendritic cells and Langerhan's cells, which are cellular sources of chemokines, such as mC10, for the recruitment of T cells, and of cytokines, which are involved in initiating inflammatory events. Mohamadzadeh does not discuss or even mention the use of chemokines generally, or mC10 specifically, as immunomodulators in immunotherapy, or

the use of mC10 as APC chemotaxin, *i.e.*, chemotactic for dendritic cells. Mohamadzadeh also does not teach or suggest that mC10 can exert biological activity in a heterologous host, *i.e.*, a primate.

#### 1.2.4 Orlofsky

Orlofsy discusses the potential role for mC10 in Th2 immune reactions. mC10 is disclosed as a chemoattractant for macrophages, and T and B cells. Orlofsky does not discuss or even mention the use of chemokines generally, or mC10 specifically, as immunomodulators in immunotherapy, or the use of mC10 as APC chemotaxin, *i.e.*, chemotactic for dendritic cells. Orlofsky also does not teach or suggest that mC10 can exert biological activity in a heterologous host, *i.e.*, a primate.

### 1.3 Appellants' Position

The Examiner has failed to establish a *prima facie* case of obviousness because there was no motivation to make the specific combination of references that would lead to the presently claimed invention. "To establish a *prima facie* case of obviousness based on a combination of the content of various references, there must be some teaching, suggestion or motivation in the prior art to make the *specific* combination that was made by the applicant." *In re Dance*, 160 F.3d 1339, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998) (emphasis supplied). The motivation must have sufficient "force" to "impel persons skilled in the art to do what applicant has done." *Ex parte Levengood*, 28 USPQ2d 1300, 1302 (BPAI 1993).

#### 1.3.1 No Teaching or Suggestion to Use Chemokines as Immunomodulators in Cancer Immunotherapy

The Examiner takes the view that Kedar's reference to cytokines would be construed as a suggestion to use chemokines because chemokines are included within the genus of cytokines. Although it is not disputed that chemokines are conventionally considered to be a species of cytokines, such a relationship is not itself sufficient to establish a suggestion to use



chemokines. To argue that mention of a genus inherently discloses all species is "wholly meritless whether considered under section 102(b) or under 103." *Corning Glass Works v. Sumitomo Electric USA Inc.*, 9 USPQ2d 1962 (Fed. Cir. 1989).

Here, there is nothing more than a bare species-genus relationship to evidence a suggestion to use chemokines. To the contrary, all of Kedar's teaching would be construed by the skilled artisan as pointing to uses of types or classes of cytokines distinct from chemokines. As discussed in detail below, there is nothing in Kedar or the other references that would have led the skilled artisan, at the effective filing date of the application, to have understood chemokines to be potentially useful as immunomodulators in cancer immunotherapy from Kedar's reference to the large genus of cytokines. A careful examination of Kedar demonstrates that the authors did not at all contemplate use of the subgenus of chemokines, or the species mC10, when considering the types or classes of cytokines in the past, present and future of cancer immunotherapy.

First, Kedar specifically mentions many cytokines, including IL-2, IFN $\gamma$ , IFN $\alpha$ , IFN $\beta$ , TNF $\alpha$ , IL-1, IL-4, IL-6, IL-7 and IL-10, and discusses several classes of cytokines that are potentially useful for cancer immunotherapy, including lymphokines, interleukins, and colony stimulating factors; however, the subgenus of chemokines are not mentioned. In fact, Kedar does not even once mention a single chemokine in almost 50 pages reviewing past, present and future methods of cancer immunotherapy.

Second, in the first full paragraph on page 258, Kedar states that "over 20 cytokines (among them 12 interleukins) are known and functionally defined." At the time Kedar was published, more than a dozen functionally defined chemokines were known in the literature (Fundamentals of Immunology, 3rd Edition, Paul, WE., ed., Raven Press, New York, 1993, pp. 822-826). Thus, it is apparent that Kedar did not mean to include the subgenus of chemokines among the number of known and functionally defined cytokines useful for cancer immunotherapy.

Third, in the last full paragraph on page 258, Kedar states that "cytokines can be useful in cancer treatment by (1) exerting direct effects on the tumor (cytolysis, cytostasis, vasculature damage, terminal differentiation), (2) enhancing expression of MHC antigens, cell adhesion molecules, and other surface moieties on the tumor cells, including tumor-associated antigens, (3) recruiting, expanding and stimulating endogenous effector cells, and (4) maintaining and even enlarging adoptively transferred lymphocyte populations." At the time Kedar was published, chemokines were well-known in the art as secreted proteins with chemotactic activity (Fundamentals of Immunology, 3rd Edition, Paul, WE., ed., Raven Press, New York, 1993, pp. 822-826). None of the above-mentioned activities of the types of cytokines thought to be beneficial for cancer immunotherapy was known or believed to be an activity associated with chemokines in general, or mC10 in particular.

Based on the foregoing, it is clear that Kedar's reference to cytokines would have been at most construed as suggesting the use of cytokines specifically mentioned therein or their equivalents, but not the subgenus of chemokines, which at the time of the instant invention was known or believed to lack the beneficial cancer immunotherapeutic activities that Kedar ascribes to cytokines.

#### 1.3.2 No Motivation to Use mC10 in Vaccines to be Administered in Primates

There was no motivation in the references or in the general knowledge in the art at the effective filing date of the application to use mC10 in vaccines to be administered to primates. The various sources of motivation asserted by the Examiner to combine the references are no more than the reasons for performing the individual methods discussed in each of the references.

The first source of motivation alleged by the Examiner for combining the references (*i.e.*, Kedar's discussion of the use of cytokines in cancer immunotherapy in general, and the advantages of using tumor antigens and cytokines encapsulated within liposomes for improving immunogenicity in particular) is the reason for doing (1) what was already being done,

namely, using cytokines for cancer immunotherapy, and (2) what Kedar proposes, namely, encapsulating a tumor antigen and a cytokine, or a combination of cytokines. The alleged motivation does not point to any specific modification of Kedar's teaching. As discussed above, Kedar does not teach or suggest the possibility of using a chemokine in place of the types of cytokines discussed. The alleged motivation particularly would not have impelled the skilled artisan to replace the types of cytokines discussed in Kedar with a chemokine, in particular mC10, alone or in combination with another chemokine in a pharmaceutical composition (*e.g.*, vaccine), as recited in claims 89 and 92.

The second source of motivation alleged to support combination of the references (*i.e.*, Bystryn's using pH sensitive liposomes to encapsulate an antigen and an immunomodulator) is likewise the reasons for doing what Bystryn was already doing, namely, using pH sensitive liposomes to improve vaccine administration and immune responses. Bystryn specifically recites cytokines, including IL-1, IL-2, IL-6, IL-12 and GM-CSF as immunomodulators, but does not even mention chemokines. The alleged motivation does not point to any particular modification of Kedar's or Bystryn's teaching, and particularly would not have impelled the skilled artisan to select a chemokine in general, or mC10 in particular, from the genus of cytokines, for use in a vaccine. Bystryn does not teach or suggest that chemokines are considered among immunomodulators useful in vaccines.

The third source of motivation alleged to support combination of the references (*i.e.*, Mohamadzadeh's teaching of dendritic cells and Langerhan's cells, which are cellular sources of chemokines, including mC10, which recruit T cells, and of cytokines, which are involved in the initiation of inflammatory events) is likewise deficient. The alleged motivation is merely the reasons for performing studies discussed by Mohamadzadeh, namely, characterizing dendritic cells as antigen-presenting cells. The alleged motivation particularly would not have impelled the skilled artisan to select a chemokine, in particular mC10, from the genus of cytokines, for use in a vaccine.

The fourth source of motivation alleged to support combination of the references (*i.e.*, Orlofsky's teaching of possible activities of mC10 in maintaining the Th2 response and recruitment of T cells) is likewise deficient. The alleged motivation is merely the reasons for performing studies discussed by Orlofsky, namely, characterizing the role of mC10 in regulating Th2 immune reactions. The alleged motivation particularly would not have impelled the skilled artisan to select a chemokine, in particular mC10, from the genus of cytokines, for use in a vaccine.

Therefore, Bystryn, Mohamadzadeh and Orlofsky all fail to compensate for the deficiency in Kedar, namely, the lack of any teaching, suggestion or motivation to select a chemokine, in particular mC10, from the genus of cytokines for a pharmaceutical composition comprising a tumor antigen.

Even assuming, *arguendo*, Kedar is read to include the subgenus of chemokines among the genus of cytokines useful as immunomodulators for cancer immunotherapy, there is nothing in the references that would have led the skilled artisan to select mC10 among the subgenus of chemokines. Without being bound to a single mechanism of action, Appellants have found that APC chemotaxins (*i.e.*, chemokines that are chemotactic for dendritic cells, in particular immature dendritic cells) have significant adjuvant activity in primates. Kedar does not mention recruitment of dendritic cells as an activity of immunomodulators useful in cancer immunotherapy, and neither Bystryn, Mohamadzadeh, nor Orlofsky mentions or suggests that mC10 has the ability to recruit dendritic cells. In particular, Bystryn does not mention mC10 at all, Mohamadzadeh only discusses mC10 as being expressed in dendritic cells, and Orlofsky only discusses mC10 as being a macrophage and T and B cell chemoattractant. Thus, the references do not teach or suggest using mC10 in vaccines, and would not have motivated the skilled artisan to select mC10, among a wide variety of chemokines, for use as an immunomodulator in cancer immunotherapy. The references do not recognize the role of mC10 in recruiting dendritic cells or other activities of mC10 that would make it useful as an immunomodulator of cancer.

### 1.3.3 Impermissible Hindsight

The Federal Circuit has emphasized the requirement for evidence of particularized motivation as a safeguard against the "tempting but forbidden zone of hindsight." *In re Dembiczak*, 50 USPQ2d 1614, 1616 (Fed. Cir. 1999). As discussed in Section 1.3.2 above, the various sources of motivation asserted by the Examiner are not particularized to the claimed invention but are simply the reasons for performing the individual studies and methods discussed in the cited references. In these circumstances, the proposed manner of combination of references, which requires that the skilled artisan recognize that the types of cytokine discussed in the references can be replaced with a specific chemokine, mC10, in the absence of any teaching or suggestion that (1) the subgenus of chemokines were considered to be equally useful as the specified cytokines in cancer immunotherapy, and (2) mC10 could exert any biological activity in primates, appears to be the result of impermissible hindsight.

### 1.4 Rebuttal of Examiner's Response

The Examiner's response to Appellants' arguments from the previous Office Action is set forth on pages 7-8 of the Final Office Action mailed January 3, 2006. The Examiner's position is that the skilled artisan would have understood that a chemokine is a pro-inflammatory cytokine and thus encompassed in the family of cytokines, and, based on results obtained with vMCK-1, chemokines can exert chemoattractant activity in heterologous hosts. The issue as to whether the skilled artisan would have considered selecting a chemokine from the genus of cytokines is addressed in Sections 1.3.1 and 1.3.2 above. The issue as to whether the ability of mC10 to trigger primate APCs is unexpected is addressed in Section 1.4.1 below.

In the Advisory Action mailed May 11, 2006, the Examiner responds to Appellants' arguments from the Final Office Action (which largely follows the position taken in Section 1.3 above). The Examiner's position is that the prior art teaches the use of non-human substances in vaccines as adjuvants and carriers; therefore, the fact that mC10 is a non-human product which functions in humans to stimulate an immune response is not surprising. In

response to Appellants' argument that the skilled artisan would not have recognized a chemokine such as mC10 would be included in the teaching of Kedar and therefore would not have been motivated to select a chemokine from the genus of cytokines, the Examiner cites *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981), stating that the test for obviousness is what the combined teachings of the references would have suggested to those of ordinary skill in the art.

#### 1.4.1 Unexpected and Surprising Results Using mC10 in Vaccines

Not only was there no motivation to combine the references in the manner alleged by the Examiner, the results obtained by Appellants are unexpected and surprising. It was unexpected and surprising that a murine chemokine, mC10, having no known human homolog, could have the ability to bind and activate a primate chemokine receptor to induce and augment an immune response to a co-administered antigen. On pages 7-8 of the Final Office Action mailed January 3, 2006, the Examiner attempted to rebut Appellants' argument that the results obtained were unexpected because the skilled artisan would not have reasonably expected a murine chemokine (*i.e.*, mC10) having no human homolog to trigger primate APCs. The Examiner refers to Saederup, which discusses the ability of mouse virus-encoded chemokine vMCK-1 to exert effects on human cells, and then states that "it appears that chemokines can exert chemoattractant activity in heterologous (*sp.*) hosts. Further, the instant claims are product claims and therefore not limited by intended use in humans." The Examiner's response fails to adequately address the issue of unexpected and surprising results.

First, the Examiner failed to provide any objective evidence or reasoning why the skilled artisan would have reasonably expected mC10 to attract or trigger *primate* APCs. Given that the majority of chemokines known at the effective filing date of the application showed strict species specificity, it would have been unreasonable to expect a murine chemokine in general, or mC10 in particular, to exert chemoattractant activity in a heterologous host, *i.e.*, a primate. Appellants' finding that mC10 could nonetheless induce and augment an immune response in primates was unexpected. Given that no human homolog to mC10 exists, Appellants' finding was also particularly surprising because the skilled artisan would not have

expected mC10 to have the ability to bind and activate any primate chemokine receptor, *i.e.*, to exert a biological activity in primates.

Second, the ability of mouse virus-encoded chemokine vMCK-1 to exert effects on human cells has little or no bearing on whether it is reasonable to expect mC10 to exert chemoattractant activity in primates. The skilled artisan would have no reason to expect that the relaxed species specificity of one mouse virus-encoded chemokine could be translated to the murine chemokine, mC10.

Third, although the instant product claims are not limited by an intended use in humans, it is entirely proper for the Examiner to consider the advantages of an invention when evaluating its obviousness. The Federal Circuit has stated that "advantages...do not properly belong in the claims, the sole function of which is to point out distinctly the process, machine, manufacture or composition of matter which is patented...not its advantages." *Preemption Devices v. Minnesota Mining & Manufacturing Co.*, 732 F.2d 903, 907 (Fed. Cir. 1984). The Federal Circuit added that "it is entirely proper, nevertheless, in evaluating nonobviousness...to take into account advantages directly flowing from the invention patented." *Id.* Here, the presently claimed pharmaceutical compositions comprising mC10 and an antigen may be used for vaccine administration to humans. The suitability of a composition to administer to a human is an inherent property of the composition irrespective of whether it is actually administered to humans. This advantage naturally follows from what is claimed, and it is entirely appropriate to consider it as evidence of nonobviousness. Because chemokines in general, and mC10 in particular, were not considered as immunomodulators useful for immunotherapy in primates, and mC10 was not known to exert any biological activity in primates, it could not have been obvious to the skilled artisan at the effective filing date of the application that administration to primates of mC10 and an antigen would result in stimulation of the immune response to the antigen.

The Examiner's response in the Advisory Action misses the point of Appellants' position regarding the unexpected and surprising results that mC10 attracts and triggers primate APCs. Appellants acknowledge that a non-human antigen, *e.g.*, mC10, can stimulate immune

responses in humans; however, the immune response would be expected to be directed against the non-human antigen. What is unexpected and particularly surprising is that administration to a primate of mC10 and an antigen stimulated the immune response to the antigen. At the effective filing date of the application, the skilled artisan could not have reasonably expected a murine chemokine lacking a human homolog to stimulate an immune response to a co-administered antigen in primates. mC10 is not analogous to an adjuvant or carrier. In vaccines, the adjuvant or carrier stimulates the immune response to the co-administered antigen, not to the adjuvant or carrier itself. There is no teaching or suggestion in the references that mC10 would have adjuvant or carrier activity. There is also no teaching or suggestion in the references that mC10 would not be expected to induce an immune response to itself. Thus, the skilled artisan would certainly not have equated mC10 with an adjuvant or carrier and, accordingly, could not have reasonably expected that administration to a primate of a vaccine comprising mC10 and an antigen would stimulate the immune response to the co-administered antigen.

1.4.2 The Skilled Artisan Would Not Have Considered Using mC10 in Vaccines to be Administered to Primates

Appellants do not disagree with the Examiner that the test of obviousness is what the combined teachings of the references would have suggested to the skilled artisan. The issue here, however, is whether the skilled artisan interested in cancer immunotherapy would have recognized that it would have been desirable or feasible to replace the types of cytokines taught by Kedar with mC10 taught by Mohamadzadeh and Orlofsky. To make this determination, one must consider whether there was any teaching, suggestion or motivation that can be gleaned from the references themselves, or from the general knowledge in the art, which would have suggested to the skilled artisan the possibility of using a murine chemokine in a vaccine for cancer immunotherapy. As discussed in Sections 1.3.1 and 1.3.2 above, there was no teaching or suggestion in the references that would have motivated the skilled artisan to select a chemokine from the genus of cytokines for use in cancer immunotherapy. As discussed in Section 1.4.1 above, there was no teaching or suggestion in the references that would have led the skilled artisan to consider using a murine chemokine in vaccines to be administered to primates. The



Examiner has failed to point to anything in the general knowledge in the art that would have motivated the skilled artisan to consider a vaccine comprising a murine chemokine in general, or mC10 in particular, and an antigen, as recited in claim 89.

**B. Issue 2: Claims 69-72, 75 and 79-88 Not Obvious Over Kedar in View of Bystryn and Saederup**

2.1 The Examiner's Rationale

The Examiner's rationale is set forth on pages 5-7 of the Final Office Action mailed January 3, 2006.

The Examiner cites Kedar as discussing cancer immunotherapy methods, and Bystryn as discussing pH sensitive liposomes, as described in Section 1.1 above.

The Examiner cites Saederup as discussing vMCK-2. According to the Examiner, Saederup teaches that: (1) vMCK-1/vMCK-2 are responsible for promoting host leukocyte chemotaxis, and may be responsible for attracting monocytes and macrophages; (2) vMCK-1 and vMCK-2 have the same chemokine domain, but vMCK-2 contains an additional 199 amino acid C-terminal domain as a result of RNA splicing; (3) vMCK-1 can recruit and activate monocytes or macrophages; and (4) a mutant lacking both vMCK-1 and vMCK-2 cannot sustain an *in vivo* inflammatory response, consistent with a role for vMCK-1 and/or vMCK-2 in maintaining monocyte migration.

According to the Examiner, it would have been obvious to use vMCK-2 as an immunomodulator in the stealth liposomes taught by Kedar, and to combine vMCK-2 with a tumor antigen, an additional chemokine and an adjuvant in the liposomes for administration to patients.

The motivation asserted by the Examiner is that Kedar teaches the accumulation of stealth liposomes at the tumor site and the therapeutic effect associated with the recruitment of monocytes and macrophages to the tumor site, and Saederup teaches recruitment of monocytes

and macrophages by vMCK-1/vMCK-2. According to the Examiner, based on the teaching of Saederup, the skilled artisan would conclude that vMCK-1 and vMCK-2 can be used interchangeably.

The motivation asserted by the Examiner to combine vMCK-2 with an additional chemokine is to exert an additive or synergistic effect. The motivation asserted by the Examiner to further encapsulate the adjuvant is the teachings of Bystryn. The motivation asserted by the Examiner to prepare sterile preparations of the liposome encapsulated pharmaceuticals is to preserve the shelf life of the pharmaceuticals and to prevent contamination.

## 2.2 Summary of the References

### 2.2.1 Kedar

Kedar is discussed in Section 1.2.1 above. Kedar does not discuss or even mention the use of chemokines generally, or vMCK-2 specifically, in cancer immunotherapy.

### 2.2.2 Bystryn

Bystryn is discussed in Section 1.2.2 above. Bystryn does not discuss or even mention the use of chemokines generally, or vMCK-2 specifically, in immunotherapy.

### 2.2.3 Saederup

Saederup discusses vMCK-1, which acts on human macrophage cells, and vMCK-1/vMCK-2, which may be responsible for attracting monocytes or macrophages to the site of viral infection. Saederup does not demonstrate that vMCK-1 and vMCK-2 have the same *in vitro* activities. Saederup does not discuss or even mention the use of chemokines generally, or vMCK-2 specifically, in immunotherapy. Saederup does not teach or suggest that vMCK-2 can exert any biological activity in primates.

## 2.3 Appellants' Position

The Examiner has failed to establish a *prima facie* case of obviousness because there was no motivation to make the specific combination of references that would lead to the presently claimed invention. The asserted motivation to combine the references is entirely predicated on the Examiner's presumption that Kedar's reference to cytokines would have been construed by the skilled artisan as a suggestion to use a chemokine in cancer immunotherapy. As discussed in detail in Section 1.3.1 above, there is nothing in Kedar or the other references supporting the Examiner's presumption, and a careful examination of Kedar demonstrates that the authors did not at all contemplate using chemokines in general, or vMCK-2 in particular, for cancer immunotherapy.

2.3.1 No Motivation to Use vMCK-2 in Vaccines to be Administered to  
Primates

There was no motivation in the references or in the general knowledge in the art at the effective filing date of the application to use vMCK-2 in vaccines to be administered to primates. The various sources of motivation asserted by the Examiner to combine the references are no more than the reasons for performing the individual methods discussed in the references.

The first and second sources of motivation for combining the references alleged by the Examiner (*i.e.*, Kedar and Bystryn) are addressed in Section 1.3.2 above. A further motivation alleged by the Examiner is that Kedar teaches the therapeutic effect associated with recruitment of monocytes and macrophages to the tumor site. The Examiner has taken this teaching of Kedar out of context. On pages 253-254, Kedar states that "regressing tumors are often infiltrated by macrophages, neutrophils and eosinophils" and that "BRM-stimulated nondiscriminative effector cells, such as macrophages and NK/LAK cells, may contribute to the therapeutic effect." The straightforward interpretation of Kedar is that the therapeutic effect of cytokines may be due to activation of macrophages already present at the tumor site. Thus, there would be no need to administer a chemokine to *attract* macrophages that have already infiltrated the tumor, whereas it would be desirable to administer a cytokine to *activate* the infiltrated macrophages and NK/LAK cells. At the effective filing date of the application, there was no

teaching or suggestion that chemokines in general, or vMCK-2 in particular, would have the ability to *activate* APCs. The alleged motivation does not point to any particular modification of Kedar's or Bystry'n's teaching, and particularly would not have impelled the skilled artisan to select chemokines in general, or vMCK-2 in particular, from the genus of cytokines, for use in a vaccine. Neither Kedar nor Bystry'n teaches or suggests that a chemokine is to be considered among those types of cytokines (*i.e.*, immunomodulators) useful in vaccines.

The third source of motivation alleged to support combination of the references (*i.e.*, Saederup's teaching of recruitment of monocytes and macrophages by vMCK-1/vMCK-2 and the alleged interchangeability of vMCK-1 and vMCK-2) does not compensate for the deficiency in Kedar. As discussed in Section 2.3.2 below, the skilled artisan would not have reasonably expected the activities of vMCK-1 and vMCK-2 to be interchangeable. Accordingly, the alleged motivation is merely the reasons for performing studies to further investigate the activities of vMCK-1 and vMCK-2. The alleged motivation would not have impelled the skilled artisan to select chemokines from the genus of cytokines, in particular vMCK-2, for use in a vaccine.

Therefore, Bystry'n and Saederup both fail to compensate for the deficiency in Kedar, namely, the lack of any teaching, suggestion or motivation to replace the types of cytokines discussed in the references with vMCK-2 in pharmaceutical compositions comprising a chemokine and an antigen.

Even assuming, *arguendo*, Kedar is read to include the subgenus of chemokines among the genus of cytokines useful as immunomodulators for cancer immunotherapy, there is nothing in the references that would have led the skilled artisan to select vMCK-2 among the subgenus of chemokines. Without being bound to a single mechanism of action, Appellants have found that APC chemotaxins (*i.e.*, chemokines that are chemotactic for dendritic cells, in particular immature dendritic cells) have significant adjuvant activity in primates. Kedar and Bystry'n do not mention recruitment of dendritic cells as an activity of immunomodulators useful in immunotherapy, and Saederup does not mention or suggest that vMCK-2 has the ability to

recruit dendritic cells. In particular, Saedeup shows that vMCK-1 has chemokine-like activities on peritoneal macrophages, but no experimental data using vMCK-2 is provided. Thus, the references do not teach or suggest using vMCK-2 in vaccines, and would not have motivated the skilled artisan to select vMCK-2, among a wide variety of chemokines, for use as an immunomodulator for cancer immunotherapy. The references do not recognize the role of vMCK-2 in recruiting dendritic cells or other activities of vMCK-2 that would make it useful as an immunomodulator of cancer.

### 2.3.2 Impermissible Hindsight

As discussed in Section 2.3.1 above, the various sources of motivation asserted by the Examiner are not particularized to the claimed invention but are simply the reasons for performing the individual studies and methods discussed in the cited references. In these circumstances, the proposed manner of combination of references, which requires that the skilled artisan recognize that the types of cytokines discussed in the references can be replaced with a specific chemokine, vMCK-2, in the absence of any teaching or suggestion that (1) the subgenus of chemokines were considered to be equally useful as the specified cytokines in cancer immunotherapy, (2) vMCK-1 and vMCK-2 were interchangeable, and (3) vMCK-2 could exert biological activity in primates, appears to be the result of impermissible hindsight.

## 2.4 Rebuttal of Examiner's Response

The Examiner's response to Appellants' arguments from the previous Office Action is set forth on pages 7-8 of the Final Office Action mailed January 3, 2006. The Examiner's position is that the skilled artisan would have understood that a chemokine is a pro-inflammatory cytokine and thus encompassed in the family of cytokines, and, based on results obtained with vMCK-1, chemokines can exert chemoattractant activity in heterologous hosts. The issue as to whether the skilled artisan would have considered selecting a chemokine from the genus of cytokines is addressed in Sections 1.3.1 and 2.3.1 above. The issue as to whether the

ability of vMCK-2 to attract and trigger primate APCs is unexpected is addressed in Section 2.4.1 below.

In the Advisory Action mailed May 11, 2006, the Examiner responds to Appellants' arguments from the Final Office Action (which largely follows the position taken in Section 2.3 above). The Examiner's position is that the prior art teaches the use of non-human substances in vaccines as adjuvants and carriers; therefore, the fact that vMCK-2 is a non-human product which functions in humans to stimulate an immune response is not surprising. In response to Appellants' argument that the skilled artisan would not have recognized a chemokine such as vMCK-2 would be included in the teaching of Kedar and therefore would not have been motivated to select a chemokine from the genus of cytokines, the Examiner cites *In re Keller*, 642 F.2d 413, 208 USPQ 871 (CCPA 1981), stating that the test for obviousness is what the combined teachings of the references would have suggested to those of ordinary skill in the art.

#### 2.4.1 Unexpected and Surprising Results Using vMCK-2 in Vaccines

Not only was there no motivation to combine the references in the manner alleged by the Examiner, the results obtained by Appellants were unexpected and surprising. It was unexpected and surprising that a murine virus-encoded chemokine, vMCK-2, having no known human virus homolog, could have the ability to bind and activate a primate chemokine receptor to induce and augment an immune response to a co-administered antigen. On pages 7-8 of the Final Office Action mailed January 3, 2006, the Examiner rebutted Appellants' argument that the results obtained were unexpected because the skilled artisan would not have reasonably expected a murine virus-encoded chemokine (*i.e.*, vMCK-2) to trigger primate APCs. The Examiner alleges that Saederup "teaches that a chemokine encoded by MCK-2 exerted an effect on cells bearing the human chemokine receptor CCR3 and the human macrophage line was also responsive to MCK-1 (abstract). Thus, it appears that chemokines can exert chemoattractant activity in heterologous (*sp.*) hosts." Appellants disagree for the following reasons.

First, the Examiner is mistaken in stating that Saederup teaches that vMCK-2 exerts an effect on human CCR3-bearing cells. On page 10884, Saederup states that "only human CCR3, a natural receptor for eotaxin, RANTES and MCP-3, was found to respond to MCK-1." Thus, vMCK-1, not vMCK-2, has been shown to exert an effect on human cells.

Second, the Examiner presumes that vMCK-1 and vMCK-2 share chemoattractant activity, based on the fact that the N-terminal chemokine domains of vMCK-1 and vMCK-2 are identical. However, given that structural and functional characterization of the C-terminal domain of vMCK-2 was lacking, it would not have been possible to predict its effect on the N-terminal chemokine domain. As such, it would have been imprudent for the skilled artisan to presume that the large C-terminal extension of vMCK-2 would not affect the N-terminal chemokine domain. In the absence of actual experimental data, the skilled artisan in the field of chemokines would not have assumed that vMCK-1 and vMCK-2 were interchangeable without further experimentation.

Given the uncertainty regarding the functional similarity between vMCK-1 and vMCK-2, it would have been unreasonable to expect vMCK-2 to exert chemoattractant activity in primates. Appellants' finding that vMCK-2 could nonetheless induce and augment an immune response in primates was unexpected and particularly surprising because there is no homolog of vMCK-2 produced by a virus that infects humans.

Third, although the instant product claims are not limited by an intended use in humans, as discussed in Section 1.4.1 above it is entirely proper for the Examiner to consider the advantages of an invention when evaluating its obviousness. Because chemokines in general, and vMCK-2 in particular, were not considered as immunomodulators useful for cancer immunotherapy, and vMCK-2 was not known to exert any biological activity in primates, it would not have been obvious to the skilled artisan at the effective filing date of the application that administration to a primate of vMCK-2 and an antigen would stimulate the immune response to the antigen.

The Examiner's response misses the point of Appellants' position regarding the unexpected and surprising results that vMCK-2 triggers primate APCs. Appellants acknowledge that a non-human antigen, *e.g.*, vMCK-2, can stimulate immune responses in humans; however, the immune response would be expected to be directed against the non-human antigen. What is unexpected and particularly surprising is that administration to a primate of vMCK-2 and an antigen stimulated the immune response to the antigen. At the effective filing date of the application, the skilled artisan could not have reasonably expected a murine virus-encoded chemokine lacking a human virus homolog to stimulate an immune response to a co-administered antigen in primates. vMCK-2 is not analogous to an adjuvant or carrier. In vaccines, the adjuvant or carrier stimulates the immune response to the co-administered antigen, not to the adjuvant or carrier itself. There is no teaching or suggestion in the references that vMCK-2 would have adjuvant or carrier activity in a primate. There is also no teaching or suggestion in the references that vMCK-2 would not be expected to induce an immune response to itself. Thus, the skilled artisan would certainly not have equated vMCK-2 with an adjuvant or carrier and, accordingly, could not have reasonably expected that administering to a primate a vaccine comprising vMCK-2 and an antigen would stimulate the immune response to the co-administered antigen.

2.4.2 The Skilled Artisan Would Not Have Considered Using vMCK-2 in  
Vaccines to be Administered to Primates

Appellants do not disagree with the Examiner that the test of obviousness is what the combined teachings of the references would have suggested to the skilled artisan. The issue here, however is whether the skilled artisan interested in cancer immunotherapy would have recognized that it would have been desirable or feasible to replace the types of cytokines taught by Kedar with vMCK-2 taught by Saederup. To make this determination, one must consider whether there was any teaching, suggestion or motivation that can be gleaned from the references themselves, or from the general knowledge in the art, that would have suggested to the skilled artisan the possibility of using a murine viral chemokine in a vaccine for cancer immunotherapy. As discussed in Sections 1.3.1 and 2.3.1 above, there was no teaching or



suggestion in the references that would have motivated the skilled artisan to select a chemokine from the genus of cytokines for use in cancer immunotherapy. As discussed in Section 2.4.1 above, there was no teaching or suggestion in the references that would have led the skilled artisan to consider using a murine virus-encoded chemokine in a vaccine preparation to be administered to primates. The Examiner has failed to point to anything in the general knowledge in the art that would have motivated the skilled artisan to consider a vaccine comprising a murine chemokine in general, or vMCK-2 in particular, and an antigen, as recited in claim 69.

## **8. CONCLUSION**

For these reasons, it is respectfully submitted that the rejection should be reversed.

Respectfully submitted,

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## **9. CLAIMS APPENDIX**

69. A pharmaceutical composition comprising viral murine cytomegalovirus chemokine 2 (vMCK-2) and an antigen.

70. The pharmaceutical composition of claim 69, further comprising a pharmaceutically acceptable excipient.

71. The pharmaceutical composition of claim 69, further comprising an adjuvant.

72. The pharmaceutical composition of claim 69, further comprising an additional chemokine.

75. The pharmaceutical composition of claim 69, wherein the chemokine and antigen are linked.

79. The pharmaceutical composition of claim 69, wherein the composition is encapsulated in a liposome.

80. The pharmaceutical composition of claim 69, wherein the composition is encapsulated in a microsphere.

81. The pharmaceutical composition of claim 69 which is sterile.

82. The pharmaceutical composition of claim 81, wherein the composition is suitable for peritoneal administration.

83. The pharmaceutical composition of claim 81, wherein the composition is formulated for administration by injection.

84. The pharmaceutical composition of claim 81, wherein the composition is formulated for administration by inhalation.

85. The pharmaceutical composition of claim 81, wherein the composition is formulated for topical application.

86. The pharmaceutical composition of claim 81, wherein the composition is formulated for oral administration.

87. The pharmaceutical composition of claim 81, wherein the composition is formulated for administration by suppository.

88. The pharmaceutical composition of claim 69, wherein the antigen is a tumor-associated antigen.

89. A pharmaceutical composition comprising mC10 and an antigen.

90. The pharmaceutical composition of claim 89, further comprising a pharmaceutically acceptable excipient.

91. The pharmaceutical composition of claim 89, further comprising an adjuvant.

92. The pharmaceutical composition of claim 89, further comprising an additional chemokine.

93. The pharmaceutical composition of claim 89, wherein the chemokine and antigen are linked.

97. The pharmaceutical composition of claim 89, wherein the composition is encapsulated in a liposome.

98. The pharmaceutical composition of claim 89, wherein the composition is encapsulated in a microsphere.

99. The pharmaceutical composition of claim 89 which is sterile.

100. The pharmaceutical composition of claim 99, wherein the composition is suitable for peritoneal administration.

101. The pharmaceutical composition of claim 99, wherein the composition is formulated for administration by injection.

102. The pharmaceutical composition of claim 99, wherein the composition is formulated for administration by inhalation.

103. The pharmaceutical composition of claim 99, wherein the composition is formulated for topical application.

104. The pharmaceutical composition of claim 99, wherein the composition is formulated for oral administration.

105. The pharmaceutical composition of claim 99, wherein the composition is formulated for administration by suppository.

106. The pharmaceutical composition of claim 89, wherein the antigen is a tumor-associated antigen.

## **10. EVIDENCE APPENDIX**

1. Fundamentals of Immunology, 3rd Edition, Paul, WE., ed., Raven Press, New York, 1993, pp. 822-826.
  - a. filed with Appellants' "Amendment After Final" dated April 3, 2006, in response to the Final Office Action, mailed January 3, 2006;
  - b. the Advisory Action mailed May 5, 2006, indicates that the Amendment After Final ("a.", above) will be entered.

**11. RELATED PROCEEDINGS APPENDIX**

None.

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# FUNDAMENTAL IMMUNOLOGY

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*THIRD EDITION*

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Editor

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suppressive and anti-inflammatory effects as well as proinflammatory and selected immunoenhancing activities. TGF- $\beta$  appears to stimulate resting cells and to suppress the same cell types if activated. TGF- $\beta$  when administered systemically acts as an inhibitor, but if given locally can promote inflammation. These bifunctional effects of TGF- $\beta$  make it difficult to predict its overall contribution to a given inflammatory host reaction.

## PDGF AND PDGF RECEPTORS

PDGF and PDGF-like growth factors for mesenchymal cells are basic 30-kD cytokines that are produced by platelet  $\alpha$  granules, endothelial cells, fibroblasts, smooth muscle cells, and macrophages (as reviewed in ref. 361). Antibodies to PDGF react with all these products and they all bind to the same receptor for PDGF. PDGF consists of two 17-kD peptide chains ("A" and "B"), with 60% sequence homology, as one of three possible isoforms (AB, AA, or BB). The biological effects of these isoforms is qualitatively similar since they each bind to the same high-affinity cell surface receptor with a  $K_D$  of  $10^{-9}$  to  $10^{-11}$  M.

The 180-kD receptor for PDGF is composed of two distinct subunits ( $\alpha$  and  $\beta$ ) that dimerize upon ligand binding: the A chain of PDGF can bind only to the  $\alpha$  subunit, while the B chain of PDGF chain can only bind to the  $\beta$  subunit. Thus the reactivity of a given cell type depends on the total number as well as ratio of  $\alpha$  and  $\beta$  receptor subunits (385). Both  $\alpha$  and  $\beta$  subunits have single transmembrane regions and their cytoplasmic portions are tyrosine kinases. Part of their signaling mechanism is to activate phospholipase C (by tyrosine phosphorylation), which then induces phosphoinositide turnover and subsequent activation of PKC (386). The PDGF receptors are homologous to the other tyrosine kinase receptors, including M-CSF receptor, c-kit, and the fibroblast growth factor receptor. A wide range of 25,000 to 150,000 receptors are expressed on a variety of cell types including macrophages, neutrophils, fibroblasts, capillary endothelial cells, and smooth muscle cells.

## PDGF ACTIVITIES

The wound-healing activities of PDGF are mediated by its mitogenicity for fibroblasts and enhanced production of fibronectin and hyaluronic acid, which are critical components of extracellular matrix and of collagenase, which is vital for scar remodeling (reviewed in ref. 385). PDGF induces IGF-1, which is partly responsible for its mitogenicity (385), and also TGF- $\beta$ , which initiates collagen production by fibroblasts (387).

PDGF is chemotactic for fibroblasts, monocytes, and neutrophils (385) and this may be mediated by its capac-

ity to induce chemokines, such as MCAF and GRO/MGSA. PDGF is also a potent activator of neutrophil granule enzyme release, superoxide anion production, and enhanced adherence. The wound-healing effects of PDGF are macrophage dependent. Administration of neutralizing anti-PDGF to rats with mesangial proliferative nephritis reduced the mesangial cell proliferation and largely prevented the deposition of extracellular matrix in the kidneys, suggesting PDGF contributes to glomerulonephritis (388). PDGF has been found at sites of inflammation and is involved in the pathogenesis of atherosclerosis (389). This may be abetted by its vasoconstrictive effects and potential for inducing hypoxia. PDGF can enhance immune responses based on its ability to augment the expression of MHC class II antigens on macrophages (390). This may be, in part, attributable to its capacity to induce IFN- $\gamma$  production by lymphocytes (391). Thus PDGF not only participates in wound repair but also indirectly enhances immune cell-mediated inflammatory reactions.

## THE CHEMOKINE FAMILY OF INFLAMMATORY CYTOKINES

Members of the family of chemotactic cytokines (Tables 7 and 8), which have been proposed to be named "chemokines" for short, are being identified as vital initiators and promulgators of inflammatory and immunological reactions (as reviewed in ref. 392). The chemokines range from 8 to 11 kD in MW, are active over a 1- to 100-ng/ml concentration range, and are produced by a wide variety of cell types. They are induced by exogenous irritants and endogenous mediators such as IL-1, TNF, PDGF, and IFN- $\gamma$ . The chemokines bind to specific cell surface receptors with a  $K_D$  of 0.4 to 4 nM. These chemokines can be considered "second-order" cytokines that appear to be less pleiotropic than "first-order" proinflammatory cytokines because they are not potent inducers of other cytokines and exhibit more specialized functions in inflammation and repair. As shown in Table 7, some of the chemokines have been assigned to a "chemokine  $\alpha$ " subset based on their gene cluster on chromosome 4 (q12-21) and based on the fact that the first two of their four cysteine groups are separated by one amino acid (C-X-C). This chemokine  $\alpha$  group includes IL-8, melanoma growth-stimulating activity (MGSA/GRO), platelet factor 4 (PF-4),  $\beta$  thromboglobulin ( $\beta$ TG), IP-10, and ENA-78. As shown in Table 8, the chemokine  $\beta$  subgroup is located on chromosome 17 (q11-32), has no intervening amino acid between the first two cysteines (C-C), and includes macrophage chemotactic and activating factor (MCAF/MCP-1), RANTES, LD-78 (also known as human MIP-1 $\alpha$ , ACT-2, or huMIP-1 $\beta$ ), and I-309 (as reviewed in ref. 393).

IL-8 is produced by many cell types including NK

TABLE 7. Properties of the chemokine  $\alpha$  subfamily

Cytokine	Cell sources	Exogenous stimulants	Endogenous inducers	Chemotactic or haptotactic responses	Major activities
IL-8	Monocytes Neutrophils Fibroblasts Endothelial cells Keratinocytes Large granular lymphs T lymphocytes	Endotoxin Mitogens Particulates Viruses	IL-1 TNF IFN- $\gamma$ costimulant IL-3	Neutrophils Basophils Unstimulated T cells Melanoma cells	Activates PMN $\uparrow$ Neutrophil adhesion $\downarrow$ Basophil histamine $\uparrow$ Keratinocyte growth Acute inflammation
GRO- $\alpha\beta\gamma$ /mu/KC muMIP-2 $\alpha\beta$	Monocytes Fibroblasts Endothelial cells	Endotoxin	IL-1 TNF	Neutrophils	Degranulates PMN $\uparrow$ Melanoma cell growth $\uparrow$ Fibroblast growth Acute inflammation
CTAP III/ $\beta$ TG	Monocytes Platelets	Platelet activators		Fibroblasts	$\uparrow$ Fibroblast growth
$\beta$ TG/NAP-2	Monocytes Platelets	Platelet activators		Neutrophils	Activates PMN
PF-4	Platelets	Platelet activators		Fibroblasts	$\uparrow$ Fibroblast growth Reverses immune suppression $\uparrow$ I-CAM1 on E.C. $\uparrow$ Chronic inflammation
IP-10/muCRG-2	Monocytes Fibroblasts Endothelial cells Keratinocytes	Endotoxin	IFN- $\gamma$	Monocytes Activated T lymphocytes	$\uparrow$ Chronic inflammation
ENA-78	Epithelial cells		IL-1 TNF	Neutrophils	Activates PMN

cells and T lymphocytes in response to exogenous stimuli such as polyclonal mitogens, injurious stimuli, and infectious agents, as well as proinflammatory cytokines such IL-1 and TNF. IL-8 is a chemoattractant of neutrophils, basophils, and a small proportion (10% or less) of resting CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes. IL-8 additionally activates neutrophil enzyme release. IL-8 is also haptotactic for melanocytes and is a comitogenic stimulant of keratinocytes.

IL-8 promotes the adherence of neutrophils to endothelial cells. IL-8 does so by inducing neutrophils to express  $\beta_2$  integrins. Neutrophils then extravasate by moving between the endothelial cell junctions and through the basement membrane to accumulate in the tissues (394). Intracutaneous injections of IL-8 cause a rapid local neutrophilic infiltration peaking within 3 hr. Intravenous administration of IL-8 does not induce systemic sequelae of elevation of acute-phase proteins or fever but does induce a neutrophilia. Intravenous administration of IL-8 also specifically reduces local peripheral inflammatory responses to IL-8, fMLP, and C5a (395). This transient anti-inflammatory effect of IL-8 probably can

be attributed to desensitization of neutrophils by systemically distributed IL-8.

Two distinct but homologous (70% at the amino acid level) receptors for IL-8 have been cloned. The IL-8 receptors are members of the rhodopsin receptor family and have a seven transmembrane spanning region (396,397). The receptors are probably coupled to G-proteins, transduce phosphoinositol hydrolysis, and are capable of rapid elevation of diacylglycerol and cytosolic Ca<sup>2+</sup> levels, which may lead to activation of protein kinase C (398). IL-8 receptors are expressed by neutrophils, which display both types of IL-8R and their expression is upregulated by G-CSF (A. Lloyd et al., *unpublished results*). Mature neutrophils express about 20,000 receptors per cell. Myelocytic lines and basophils express several thousand receptors per cell.

A murine homologue of human IL-8 has not been identified as yet, but IL-8-like molecules have been identified in rabbits, sheep, and other species. IL-8 is in the circulation of patients with systemic inflammatory reactions or severe trauma. IL-8 has readily been detected in inflammatory sites such as in the synovial fluid in rheu-

TABLE 8. Properties of the chemokine  $\beta$  subfamily

Cytokine	Cell source	Exogenous stimulants	Endogenous stimulants	Chemotactic or haptotactic responding cells	Major activities
MCAF/MCP-1/muJE	Monocytes Fibroblasts Endothelial cells Keratinocytes Mesangial cells	Endotoxin Mitogens Particulates Bacteria	IL-1 TNF PDGF IFN- $\alpha$	Monocytes Basophils	Macrophage activation Basophil histamine release Chronic inflammation
RANTES	T lymphocytes Platelets	Mitogens Anti-CD3		Monocytes T lymphocytes (memory subset) Eosinophils Basophils	$\uparrow$ T cell/HUVEC adhesion Basophil histamine release Chronic inflammation
LD-78/muMIP-1 $\alpha$	T lymphocytes Monocytes	Mitogens Anti-CD3 Endotoxin	TNF	Monocytes Activated T lymphocytes (CD8 subset) (eosinophils)	$\downarrow$ BM progenitor stem cells Costimulates myelopoiesis Activates CD8 lymphocytes $\uparrow$ CD8 adhesion to HUVEC
ACT-2/muMIP-1 $\beta$	T lymphocytes Monocytes	Mitogens Anti-CD3 Endotoxin	TNF IL-2	Monocytes Activated T lymphocytes (CD4 subset)	Costimulates myelopoiesis Activates CD4 lymphocytes $\uparrow$ CD4/HUVEC adhesion
I-309/TCA-3	Cultured T lymphocytes	Mitogens		Monocytes	

matoid arthritis (399), extracts of psoriatic skin (400), and in the circulation of patients in septic shock (401). Thus IL-8 is implicated as a major participant in acute as well as more prolonged inflammatory reactions.

MGSA, as its name implies, was first discovered as a factor that accelerated the growth of melanoma cell lines and also as a product of oncogene transfected cell lines (GRO). MGSA/GRO competes for the type II, but not type I, IL-8 receptor on myelocytic cells (402) and is also a potent chemoattractant, as well as activator of neutrophils. MGSA as well as IL-8 has been extracted from psoriatic tissues (403).

GRO has three variants ( $\alpha$ ,  $\beta$ , and  $\gamma$ ), which exhibit about 95% homology in their amino acid sequences. They are probably homologues of murine macrophage derived KC, macrophage inflammatory peptides MIP-2 $\alpha$  and MIP-2 $\beta$ . Murine MIP-2 $\alpha$  and MIP-2 $\beta$  both compete with equal affinity for type II receptors for IL-8 and chemoattract human as well as murine neutrophils (402). MIP-2 is also reported to degranulate murine neutrophils, resulting in the release of lysosomal enzymes. Local *in vivo* injections of MIP-2 results in neutrophil accumulation and MIP-2 has been isolated from sites of wound healing. MIP-2 is a costimulator of hematopoietic colony formation by CSF-1 and GM-CSF, but the *in vivo* relevance of this observation remains to be established. It is most likely that GRO/MIP-2 inflammatory

activities overlap considerably with those of IL-8, and GRO is therefore probably also a major inflammatory mediator.

PF-4 and CTAP III, the precursor of  $\beta$ TG, are both present in platelet granules and are released by inducers of platelet aggregation. Consequently, they become available at sites of injury, hemorrhage, and thromboses. Both are reported to chemoattract and to stimulate fibroblasts, presumably for repair purposes. In addition, a 70 amino acid breakdown product of  $\beta$ TG known as neutrophil attracting peptide 2 (NAP-2) is a chemoattractant and activator of neutrophils, albeit at 100-fold higher concentrations than IL-8. NAP-2 also competes for the type II IL-8 receptor with about one-hundredth of the affinity of IL-8 (404). However, since at the site of platelet aggregation, high levels of NAP-2 can be released, it is thought to be an active participant in attracting inflammatory cells to such sites.

ENA-78 is the most recently cloned member of the chemokine  $\alpha$  subfamily (405). ENA-78 is produced by an epithelial cell line in response to IL-1 and TNF. In cross-desensitization experiments, ENA-78 also utilizes the type II receptor for IL-8 and GRO and is a chemoattractant and activator of neutrophils.

IP-10 is produced by macrophages, endothelial cells, and keratinocytes in response to IFN- $\gamma$ . The pathophysiological functions of IP-10 remain unclear, but antibod-

ies to IP-10 react with many cell types present at sites of delayed hypersensitivity reactions and IP-10 has been extracted from psoriatic plaques (406). Thus IP-10 can presumably be produced by many cell types and probably participates in chronic inflammation and delayed hypersensitivity responses. A stable recombinant human IP-10 was recently produced by Dr. K. Matsushima (*personal communication*). We have shown that this rhIP-10 is a moderately potent *in vitro* chemoattractant of human monocytes, but not neutrophils. In addition, this IP-10 also is a moderately potent chemoattractant for previously activated CD4 and CD8 T lymphocytes and promotes adhesion of lymphocytes to endothelial cells (D. Taub et al., *unpublished results*). These observations predict that IP-10 will probably be a participant in chronic cell-mediated inflammatory reactions.

Recombinant human and murine members of the chemokine  $\beta$  subfamily have become available for studies only recently. MCAF, otherwise known as MCP-1, is produced by monocytes, fibroblasts, and endothelial cells in response to the usual exogenous stimuli, as well as endogenous cytokines such as IL-1, TNF, and PDGF (392,393). MCAF chemoattracts and activates monocytes to release enzymes and become cytostatic for tumor cells. There are no detectable binding sites for MCAF on neutrophils, lymphocytes, or other cell types except monocytes. MCAF regulates the expression of surface adhesion molecules such as integrins, ELAM-1, and CD11c and b on monocytes (407). MCAF has been detected at inflamed atheromatous lesions in blood vessel walls and in the alveolar fluid of patients with pulmonary pathoses. MCAF induces macrophages to accumulate by 6 to 18 hr at sites of injection. Fibrosarcoma tumors that are infiltrated with monocytes have been shown to produce MCAF and to grow more slowly than noninfiltrated tumors. Tumor cells transfected to express MCAF also grow less well *in vivo* than their untransfected counterparts. In addition, MCAF is a potent and rapid degranulator of basophils, resulting in histamine release (408). These data suggest that basophils express MCAF receptors and may play an important role as a late histamine releasing factor (HRF) in the pathogenesis of the late phase of allergic disorders such as atopic food allergies, asthma, and chronic urticaria.

I-309, a product of activated T cells, was recently identified to have chemoattractant activity for monocytes (409). No other biological activities have been reported for I-309 to date.

RANTES is a moderately potent chemoattractant for monocytes and a very potent chemoattractant for memory T cells but not for naive T cells (410). RANTES peptides are produced by activated T lymphocytes and by platelets. Activated T cells respond to a greater extent to RANTES than unstimulated T cells. RANTES has also been detected at sites of atheromatous inflammation. RANTES promotes the adherence of T cells to hu-

man vascular endothelial cells (HUVEC) (A. Lloyd et al., *unpublished results*). This response is more marked when anti-CD3 activated T cells as well as IL-1 prestimulated human endothelial cells are used. In addition, RANTES-like MCAF causes rapid basophil degranulation and histamine release and may participate in the late phase of allergic reactions (411).

LD-78 (human MIP-1 $\alpha$ ), the homologue of murine MIP-1 $\alpha$ , has recently been shown to be an *in vitro* chemoattractant for human monocytes and activated T lymphocytes, with a preference for the CD8 subset of T cells (D. Taub et al., *unpublished results*). In addition, huMIP-1 $\alpha$  promotes the adhesion of activated CD8 lymphocytes to HUVEC (A. Lloyd et al., *unpublished results*) and also can chemoattract B lymphocytes and eosinophils. ACT-2, which is the human homologue of murine MIP-1 $\beta$ , like MIP-1 $\alpha$  chemoattracts monocytes but preferentially attracts activated CD4 rather than CD8 T cells. Similarly, human MIP-1 $\beta$  promotes the adherence of activated CD4 cells to HUVEC. Purified natural MIP-1 $\alpha$  and MIP-1 $\beta$  is reported to activate macrophages to be cytotoxic for tumor targets, to secrete TNF, IL-6, and IL-1 $\alpha$ , and in the case of mature tissue macrophages to proliferate (412). However, MIP-1 $\alpha$  and MIP-1 $\beta$  did not induce an oxidative burst or increased Ia expression by macrophages (412).

Human MIP-1 $\alpha$  and MIP-1 $\beta$  show 70% homology in their amino acid sequence. Despite this difference, both MIP-1 $\alpha$  and MIP-1 $\beta$  bind with equal affinity to monocytes and T lymphocytes and compete equally well for binding sites on these cell types. However, muMIP-1 $\alpha$  is reported to inhibit hematopoietic stem cell replication, while muMIP-1 $\beta$  not only fails to do so but competitively inhibits this activity of MIP-1 $\alpha$  (411). Some of the chemokine  $\beta$  ligands utilize the same receptors. Both MIP-1 $\alpha$  and MIP-1 $\beta$  compete for about 25% of the MCAF binding sites on monocytes. Conversely, MCAF competes for about 30% of the MIP-1 $\alpha$  and MIP-1 $\beta$  binding sites on monocytes, but not for those on lymphocytes (J. M. Wang et al., *unpublished results*). Thus there are shared and unique receptors for the MCAF and MIP-1 $\alpha$  chemokines. Although RANTES does not compete for MIP-1 $\alpha$ ,  $\beta$  or MCAF binding sites, an excess of MIP-1 $\alpha$ , MIP-1 $\beta$ , and MCAF unidirectionally partially inhibited the RANTES binding sites on macrophage cell lines. These data are supported by experiments showing that the capacity of RANTES to induce calcium influx into these cells can be inhibited by prior incubation with desensitizing doses of MIP-1 $\alpha$  and MIP-1 $\beta$  or MCAF, but not the reverse. This suggests that some of the chemokine receptors bind multiple ligands and some of the chemokines bind to multiple receptors.

Overall, the chemokine family members appear to be very potent and pivotal chemoattractants and activators of inflammatory cells and fibroblasts. However, their contribution to immune reactions are still incompletely

defined. The facts that a number of the chemokines are T cell derived, such as RANTES, MIP-1 $\alpha$ ,  $\beta$ , and I-309, and four of them (RANTES, MIP-1 $\alpha$ ,  $\beta$ , and IP-10) chemotact T lymphocytes as well as monocytes suggest that they have a significant immunological role.

## CONCLUSION

In conclusion, the aforementioned inflammatory cytokines have myriad effects on cell growth and differentiation. As indicated, some of these effects are indirect and based on the capacity of cytokines to induce the production of a cascade of other cytokines. For example, although TNF and IL-1 exhibit antiviral effects, these are mediated by IFN- $\beta$  (194). Many reports demonstrate that cytokines not only interact sequentially (one inducing another) but also reinforce one another. For example, TNF not only induces IL-6 and IL-1, but both IL-6 and IL-1 can augment the many biological effects of TNF. Furthermore, IL-1 can even induce IL-1. The interactions become even more complex when one considers the observations that IL-1 and IFN modulate not only the production of other cytokines but can also modulate the expression of functional receptors for cytokines.

All the aforementioned complex *in vitro* interactions of cytokines make it virtually impossible to predict the *in vivo* activities of a given cytokine. This is amply illustrated by the unexpectedly broad spectrum of *in vivo* activities of cytokines such as IL-1, TNF, TGF- $\beta$ , and IL-6. The actual physiological role of most of the cytokines remains to be established. In fact, we have to relearn the trite but true import of "*in vivo veritas*." As pure recombinant cytokines of each type become available, it is important to analyze the effects of excess levels of these cytokines on *in vivo* physiological and pathophysiological processes. On the other hand, depleting the cytokines, to determine what vital roles they play, is equally important; this will involve the development of effective reagents (antibodies to cytokines and receptors, soluble receptors, and other specific inhibitors produced by rational drug design) and gene targeting. The cytokines that have been knocked out to date have yielded unexpected results, generally showing less pathological consequences than anticipated.

The advantage of having multiple biochemically distinct cytokines such as IL-1 $\alpha$  and IL-1 $\beta$ , TNF and LT, TGF- $\beta$ 1,2,3, and the IFN- $\alpha$ , $\beta$  family of factors that react, within each family, with the same receptor remains to be established. Despite their apparent similarities, each may serve a unique function, as evidenced by the impact of knocking out just one of the TGF- $\beta$ s. Perhaps the existence of biochemically different ligands with the same biological activities results in advantageous differences in expression, post-translational processing, *in vivo*

half-life, tissue distribution, and access to target cells. The advantage of the apparent redundancy of cytokines, each acting on its own receptor, is also puzzling. For example, IL-1 and TNF and to a lesser extent IL-6 exhibit a broad spectrum of overlapping activities. Perhaps ligands that employ different postreceptor signal transduction pathways when acting together can activate cells at much lower concentrations. This would account for the observed synergistic effects of these cytokines. Of course, the usual reasons for redundancy such as the security of possessing alternative pathways would also be advantageous. Despite the recent explosive progress in cytokine research, there exist a number of gaps, particularly in their intracellular mechanism of action.

Therapeutic usefulness of the cytokines and their inhibitors is growing and should accelerate. We now appreciate the difficulties of systemic administration of the cytokines and it remains a challenge to harness the power nature has invested in these molecules.

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